

## Two high-amylose maize starches with different amounts of resistant starch vary in their effects on fermentation, tissue and digesta mass accretion, and bacterial populations in the large bowel of pigs

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Four groups of young pigs ( $n$  6) were fed a diet containing 50% maize starch as either a highly digestible waxy starch (control; 0% amylose) or one of three resistant starch (RS) diets, namely a high-amylose maize starch (HAMS; 85% amylose), this starch subjected to hydrothermal treatment (HTHAMS; 85% amylose), or a blend of HAMS and HTHAMS included in equal amounts, for 21 d. Food intake and live weight at the end of the study were similar among the four groups. Ileal starch digestibility was lower in pigs fed the three RS diets but was greater for HAMS (88%) than for HTHAMS (70%;  $P < 0.05$ ). Faecal output and large bowel digesta mass, and concentrations and pools of individual and total SCFA were higher (by about two- to threefold; all  $P < 0.05$ ) and digesta pH lower (by about 1 unit, all  $P < 0.001$ ) in pigs fed either HAMS or HTHAMS compared to the controls. These differences in biomarkers were seen along the length of the large bowel. Colon length was 0.5–0.9 m longer (19–35%) in pigs fed the high-RS diets relative to those fed the highly digestible starch diet ( $P < 0.05$ ). Faecal and proximal colonic lactobacilli and bifidobacteria numbers were higher (by 1 and 3 log units;  $P < 0.05$ ) in pigs fed the HAMS or HTHAMS diets. Although both high-amylose starches promoted fermentation throughout the large bowel, the data suggest that the effects of HTHAMS may be more pronounced in the distal region compared to those of HAMS.

### Resistant starch: Fermentation: Maize: SCFA: Pigs

Resistant starch (RS), the fraction of ingested starch that escapes from the small intestine (Asp, 1992), is emerging as a protective agent against several serious pathologies of the large bowel (Topping & Clifton, 2001). There is also some evidence that RS may be more effective than dietary fibre in reducing risk of colorectal cancer (Cassidy *et al.* 1994). Although the mechanisms by which RS could promote colonic health are not understood fully, the general consensus is that it acts indirectly through products of its fermentation by the gut microflora. Starch that enters the large bowel is normally actively metabolised by the numerous saccharolytic bacteria inhabiting that region of the gut (Pryde *et al.* 2003), resulting in greater SCFA production and a concomitant reduction in luminal pH (Cummings *et al.* 1996). Acidification of the intracolonic environment is considered important for suppressing the production and activity of a range of bacterial metabolites implicated in colonic disease (Topping & Bird, 1999; Bird *et al.* 2000a). SCFA also have important nutritional and physiological roles in maintaining the integrity of the bowel wall. Butyrate, one of the principal colonic SCFA, is attracting considerable interest because of its potential to lessen the risk of carcinogenesis (Brouns *et al.* 2002). In addition to modulating microbial metabolic activity, certain RS have a selective action on the bacterial composition of the large bowel

microflora which also may have health implications for the host. Studies in pigs (Brown *et al.* 1997) and mice (Wang *et al.* 2002) have shown that high-amylose maize starches (HAMS), the RS investigated most extensively thus far, are very effective in raising colonic bifidobacteria numbers. These prebiotic actions are comparable to those of established prebiotic oligosaccharides, notably the inulin-type fructans (Roberfroid, 2001).

Small intestinal starch digestibility is altered by the relative proportions of amylose and amylopectin in the granule and also by partial hydration at elevated temperature for a period of time (Sievert & Pomeranz, 1989; Kweon & Shin, 1997; Perera *et al.* 1997). HAMS is particularly susceptible to this hydrothermal (or heat-moisture) process with suggestions that changes are occurring in the associations between starch chains in the amorphous regions and the manipulations in the degree of crystalline order. The changes are intragranular and after hydrothermal treatment the intact starch granules remain. For HAMS it has been observed that hydrothermal treatment leads to increases in RS (Sievert & Pomeranz, 1989) and dietary fibre (Crosby, 2003).

The aim of the current experiment was to compare the effects of untreated HAMS with the same starch after it had been subjected to hydrothermal treatment (designated HTHAMS) on

**Abbreviations:** HAMS, high-amylose maize starches; RS, resistant starch.

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large bowel fermentation events and microbiology in pigs. This HAMS is a commercially rich source of dietary fibre and RS, with an amylose content of 85 %, and it was expected that the hydrothermal treatment would lower its small intestinal digestibility. A conventional waxy maize starch (amylose content 0 %) was used as the reference starch because of its very high small-intestinal digestibility and low RS content (Bird *et al.* 2000b; Brown *et al.* 2003). Pigs were used for the experiment as they are considered to be a good model of gut function in man (Topping & Clifton, 2001).

## Methods

### Animals

Twenty-four, 4-week-old Large White crossbred pigs (males *n* 13, females *n* 11) were used, with an initial live weight of 23.7 (SEM 0.6) kg when acquired from a local commercial piggery. They were housed individually in galvanised-steel pens with concrete floors and maintained at a controlled temperature (25 °C) for the duration of the study. Before the study commenced, the pigs were habituated to their new environment and feeding routine over 10 d during which time they were all fed the control diet (see Table 1). The study protocol was approved by the Animal Experimentation Ethics Committee of the CSIRO Health Sciences and Nutrition and conformed to published guidelines (National Health & Medical Research Council, CSIRO & Australian Agricultural Council, 1985).

### Experimental design, diets and feeding procedures

The pigs were weighed at the end of the 10-d adaptation period and assigned to one of four dietary treatment groups of six animals each on the basis of gender and live weight. It was necessary to allot unequal numbers of male and female pigs to one group selected at random (four and two respectively; HAMS + HTHAMS diet). Experimental diets were formulated to provide (by weight, as is basis): 150 g/kg protein, 540 g/kg starch, 200 g/kg lipid, and a total of 75 g/kg dietary fibre (as NSP). Diets were identical in composition except for the source of maize starch, which was either a conventional low-amylose cornstarch (Mazaca 3401C, Control) or one of two different high-amylose maize starches (HAMS or HTHAMS). The diet

for the remaining treatment group contained both high-RS ingredients, which were present in equal proportions (HAMS + HTHAMS). All three starch products were commercial food ingredients manufactured by Starch Australasia (Lane Cove, NSW, Australia). HTHAMS was prepared using HAMS under conditions where the starch had a moisture content of 25 % and it was heated to 125 °C for 120 min. HAMS and HTHAMS contain 85 % and either 30 % or 60 % total dietary fibre, respectively (Brown, 2004), whereas the starch used in the control diet had an amylose content of about 0 %. When formulating the diets allowance was made for the small amount of wheat starch (about 7 %) contributed to the whole diet by the fibre source (wheat bran, which contained 220 g starch/kg). Control and high-RS treatment diets were fed as two meals of identical weight, at 09.00 and 16.00 hours, at a rate proportional to the metabolic weight of the pigs (70 g/kg live weight<sup>0.75</sup>). Powdered rations were placed in feed hoppers and an equivalent amount of water added immediately. Pigs were weighed twice weekly prior to their morning feeding and food intake adjusted accordingly. Diets were manufactured on a weekly basis and stored in large bins at room temperature (< 20 °C) until fed. Pigs had unrestricted access to tap water for the entire period of study. Chromic oxide, a particulate digesta marker, was incorporated (0.1 g/100 g) into each diet for the final 4 d of the treatment period to determine starch digestibility. Experimental diets were fed for 21 days and the pigs then anaesthetised and killed to enable collection of tissues and digesta.

### Sampling procedures

Faeces (24-h collections) were removed from the pen floor periodically during the adaptation period and at the end of the first and second week of the treatment period and total daily faecal output was recorded. Freshly voided stools were used for bacterial enumeration and the remaining portions, after recording pH, were stored frozen to await determination of SCFA and other analytes. At the completion of the feeding period and approximately 16 h after the pigs had been fed the previous evening, they were weighed, sedated with ketamine (Ketapex; Apex Laboratories, St Mary's, NSW, Australia) and anaesthetised by inhalation of halothane (Rhone Merieux, West Footscray, VIC, Australia); mixed with O<sub>2</sub> at 4 % v/v. The procedures used for collection of plasma and digesta

**Table 1.** Composition (g/kg) of the experimental diets

Ingredient	Control	HAMS	HTHAMS	HAMS + HTHAMS
Mazaca*	515			
HAMS		515		258
HTHAMS			515	258
Casein†	122	122	122	122
Sucrose	13	13	13	13
Corn oil	192	192	192	192
Wheat bran‡	156	156	156	156
Vitamins and minerals§	2	2	2	2

HAMS, high-amylose maize starch; HTHAMS, hydrothermally treated HAMS.

\* Mazaca 3401x cornstarch supplied by Starch Australasia Ltd, Lane Cove, NSW, Australia.

† Casein obtained from Cottee Nutritionals Pty Ltd, Gordon, NSW, Australia.

‡ Wheat bran ('Clean Bran') obtained from Goodman Fielder Mills (Allied Flour and Bakery Services), Mile End, SA.

§ Standard pig grower vitamin and mineral mix obtained from Roche Vitamins Australia Ltd, Wagga Wagga, NSW, Australia.

have been described in detail previously (Bird *et al.* 2000b). In brief, the abdominal cavity was opened by midline laparotomy and an aliquot of urine withdrawn from the bladder, transferred to a labelled vial and stored frozen ( $-20^{\circ}\text{C}$ ) until analysis. The pigs were then killed by barbiturate overdose (Lethabarb, Virbac, Peakhurst, NSW, Australia) and the gastrointestinal tract immediately ligated at the oesophagus and rectum and the entire gastrointestinal tract together with the attached liver excised. The large and small bowel were isolated by means of appropriately positioned ligatures then separated and their respective unstretched lengths measured. Samples of ileal and mid colonic tissue were excised immediately and processed for histomorphometry. Small intestinal contents were removed from the distal 1 m, weighed and stored frozen ( $-20^{\circ}\text{C}$ ) for subsequent analysis. The caecum and colon were separated, and the latter divided into three segments of equal length (proximal, mid and distal colon). Digesta from each of the segments and from the caecum were extruded and weighed. Using aseptic techniques, aliquots of digesta from the proximal colon were serially diluted, the suspensions plated on appropriate differential and selective media and subsequently enumerated for key enteric bacteria. pH was recorded for the remaining portion of the digesta samples which were prepared for later analysis and stored frozen ( $-20^{\circ}\text{C}$ ). The colon and caecum, devoid of contents, were blotted dry and weighed. Frozen samples were thawed at  $4^{\circ}\text{C}$  prior to processing for analysis. Four pigs were killed each day, one from each treatment. The experiment was staggered to ensure that the experimental period (21 d) was the same for all pigs. Representative samples of meals fed to each pig over the last 4 d of study were collected and stored to await analysis of starch and chromium.

#### Biochemical analyses

Digesta, faeces and food samples were thawed at  $4^{\circ}\text{C}$ , homogenised, freeze-dried and then milled (1 mm screen) for measurement of dry matter, starch and chromium. Moisture content was calculated as the loss in mass of digesta after freeze-drying. Chromium content of digesta was determined by atomic absorption spectroscopy (Pearson & Mallett, 1972). In brief, samples were accurately weighed into an acid-washed beaker and ashed in a muffle furnace at  $250^{\circ}\text{C}$  for 1 h, followed by 4 h at  $450^{\circ}\text{C}$ . Residues were cooled overnight before adding 2.4 ml of conc.  $\text{HNO}_3$  and heating to about  $120^{\circ}\text{C}$ . Then 300 mg of potassium chlorate was added to each sample and heated until the volume had been reduced to approximately 1.6 ml. Residues were allowed to cool, diluted appropriately and analysed using an atomic absorption spectrophotometer (Varian Spectra Model AA 400, Varian, Inc.). A reducing nitrous oxide-acetylene flame in a matrix of 4%  $\text{HNO}_3$  (v/v) was used to eliminate possible interferences with Cr detection.

Total starch was analysed as free glucose after  $\alpha$ -amylase/amyloglucosidase digestion using a commercial procedure (Total Starch Assay Kit, Megazyme Ltd, Melbourne, Australia) that was based on the method of McCleary *et al.* (1994).

For SCFA analysis, aliquots of thawed large bowel contents and faeces (approximately 2 g) were mixed with 3 volumes of internal standard (3.52 mmol/l oenanthic acid) and the pH of the suspension determined using an appropriate glass

electrode. The suspension was then centrifuged for 15 min at  $3000g$  and  $4^{\circ}\text{C}$  and an aliquot of the supernatant distilled at low temperature (Vreman *et al.* 1978). Approximately 10  $\mu\text{l}$  distillate was assayed for individual SCFA by gas chromatography using a Hewlett-Packard gas chromatograph (Model HP5710) equipped with a Zebron ZB FFAP glass capillary column (0.53 mm i.d.  $\times$  30 m; Phenomenex, Pennant Hills, NSW, Australia). Individual SCFA were quantified using flame-ionisation.

#### Histomorphometry

The lumen of intestinal segments were flushed free of residual digesta using phosphate-buffered saline ( $4^{\circ}\text{C}$ , pH 7.4) and cut longitudinally along the wall adjacent to where the mesenteric border was attached. Ileal and colonic segments (about 1 cm) were excised immediately and fixed in neutral buffered formalin for 24 h and then placed in 70% ethanol (v/v) to await histological assessment. Tissue slices (approximately 1–2 mm) were cut and encased in tissue cassettes before being prepared using an automated process that involved dehydrating in ethanol, clearing with histolene, and then permeating and embedding with paraffin. Sections were cut and stained with haematoxylin and counterstained with eosin yellow. Dimensions of intestinal anatomical components were measured using light microscopy combined with computer-aided image analysis (Video Pro, Leading Edge, Bedford Park, South Australia).

#### Bacteriology

Aliquots (10 g) of freshly collected digesta from the proximal colon and faeces were serially diluted (10-fold dilutions), and 0.1 ml of each of the suspensions plated onto the following semiselective agar plates: Columbia blood medium, chromogenic *E. coli*/coliform medium and Rogosa medium (all from Oxoid, West Heidelberg, Victoria), and bifidus blood medium (Reuter, 1985), as modified by Pachinari *et al.* (2002) to enumerate total anaerobes, total aerobes, *E. coli* and coliforms, lactobacilli, and bifidobacteria, respectively. Plates were then incubated aerobically, or anaerobically using the Anaerocult A mini procedure (Merck Pty Ltd, Kilsyth, VIC, Australia), at  $37^{\circ}\text{C}$  for 1 to 6 d, depending on the assay. Relevant bacterial colonies on culture dilution plates were counted visually and the number of organisms calculated as colony forming units (CFU) per gram of wet digesta or faeces.

#### Calculations and statistics

Digesta SCFA pools were calculated as the product of concentration (mmol/l) of individual acids, or their sum (total SCFA),  $\times$  volume of digesta water (ml) in respective intestinal compartments. Faecal SCFA excretion was calculated similarly. Intestinal starch digestibility was calculated according to a standard formula (marker ratio method) using values for Cr content of feed, ileal and distal colonic digesta, and faeces. Statistical evaluations were performed using the General Linear Model (GLM) procedure of SAS version 8.02 (SAS Institute, Cary, NC, USA). Gender and block (day 0 weight class) were not significant effects and were subsequently removed from the model. Dietary treatment effects

were analysed by ANOVA. If a significant *F* test was observed ( $P < 0.05$ ), differences among means were then determined by the protected difference (PDIFF) procedure. Repeated measures ANOVA was used to analyse differences in SCFA and other fermentation-dependent measures among sampling sites in the large bowel. Differences between treatment means were considered significant at  $P < 0.05$ . For digesta and faecal counts of bacteria, the data were transformed ( $\log_{10}$ ) before statistical analysis of treatment effects. For samples that contained bacterial populations below the assay level of detection, an appropriate arbitrary value (1 log unit lower than the specified detection level for the respective assay) was assigned and used in subsequent statistical calculations. Data are shown as least squares mean and pooled standard error of the least squares mean (SEM). For bacterial counts, results are expressed as  $\log_{10}$  CFU/g. For most variables, means are for six observations per group; however, on some occasions samples (e.g. digesta) were either unavailable or present in insufficient quantities to permit analysis. For these variables, the number of observations is shown in parentheses. For tabulated results, values within a column with different superscripts differ significantly from the control.

## Results

### Food intake and body weight gain

All diets were well accepted and meals were consumed promptly. The pigs grew with an average gain of 391 (pooled SEM 13,  $n$  24) g/d for all groups across the treatment period. Final live weight (34.8 (pooled SEM 0.7) kg) and feed conversion efficiency (436 (pooled SEM 12) g/kg) were unaffected by diet but live weight gain for the treatment period was lower ( $P < 0.05$ ) for pigs fed HAMS compared to the control group (7.6 v. 9.3 (pooled SEM 0.5) kg).

### Small and large intestinal starch digestibilities

There was almost complete disappearance of starch in the gastrointestinal tract of pigs fed the control starch as evidenced by the small amount of starch in faeces ( $< 2$  g/100 g) over the whole experiment. Replacement of the control maize starch with HAMS in the treatment groups resulted in a significant increase in faecal starch concentration and excretion. For instance, in week 2, faecal starch output was 1, 30, 70 and 50 g/d for control, HAMS, HTHAMS and the HAMS + HTHAMS groups, respectively (pooled SEM 10 g/d; values all significantly different from one another).

Only very small amounts of starch were detected in ileal digesta of control pigs (Table 2). By contrast, feeding HAMS diets produced significantly higher starch levels in ileal digesta, particularly for pigs fed HTHAMS either alone or in combination with HAMS ( $P < 0.001$  and  $P < 0.05$ , respectively). Ileal starch digestibility to the terminal ileum was  $> 96\%$  in pigs fed the control diet but more than 12% and 30% of starch in the HAMS and HTHAMS diets, respectively, escaped into the large bowel (Table 3). Although the difference in ileal starch digestibility between control and groups fed high-amylose starches was large, it was not statistically significant for HAMS ( $P > 0.05$ ). These data are based on

**Table 2.** Starch content (g/100 g dry matter) of diets and of ileal and distal colonic digesta of pigs fed the experimental diets\*

(Values are least squares means and pooled SEM of six animals per group except ileum control and HTHAMS ( $n$  4) and ileum HAMS and HAMS + HTHAMS ( $n$  2))

Dietary group	Diet	Ileum	Distal colon
Control	45.8	5.5 <sup>a</sup>	2.6 <sup>a</sup>
HAMS	45.3	11.6	15.4
HTHAMS	43.5	28.6 <sup>c</sup>	26.3 <sup>c</sup>
HAMS + HTHAMS	47.7	26.9 <sup>b</sup>	32.7 <sup>d</sup>
Pooled SEM	–	4.8	5.1

HAMS, high-amylose maize starch; HTHAMS, hydrothermally treated HAMS. \*Mean values for treatment were significantly different from control: <sup>ab</sup> $P < 0.05$ , <sup>ac</sup> $P < 0.01$ , <sup>ad</sup> $P < 0.001$ .

For distal colonic digesta starch content, control v. HAMS,  $P = 0.091$  and HAMS + HTHAMS v. HAMS,  $P = 0.027$ .

a small sample size because the ileum of some pigs at slaughter was devoid of contents (see Table 3).

Very small amounts of starch were recovered from the distal colon of control pigs in contrast to those fed the RS diets (Table 2). For pigs fed HTHAMS and HAMS + HTHAMS, starch accounted for about 30% of dry matter in the distal colon, whereas for pigs fed HAMS, only about half this amount of starch was present ( $P > 0.05$ ). Nearly all of the starch that entered the colon of control pigs was fermented to completion (99.6%) whereas there was proportionately less microbial breakdown of starch in the colon of pigs fed HTHAMS or HAMS + HTHAMS (both  $P < 0.01$ ).

### Large bowel morphology

Consumption of the HAMS diets had a pronounced trophic effect on the large bowel. Although caecal weight of pigs fed the various high-RS diets was greater than that of control pigs, only in the case of HAMS were the differences statistically significant ( $P < 0.05$ ; Table 4). The colon of pigs fed the HAMS diets was also substantially heavier as well as longer relative to controls, regardless of whether the data are expressed in absolute terms (Table 4) or normalised to live

**Table 3.** Ileal and distal colonic starch digestibility (%) of pigs fed the experimental diets\*

(Values are least squares means and pooled SEM of six animals per group except ileum control and HTHAMS ( $n$  4) and ileum HAMS and HAMS + HTHAMS ( $n$  2))

Dietary group	Ileum	Distal colon
Control	96.2 <sup>a</sup>	99.6 <sup>a</sup>
HAMS	87.8	93.5
HTHAMS	69.7 <sup>c</sup>	83.2 <sup>c</sup>
HAMS + HTHAMS	73.5 <sup>b</sup>	84.7 <sup>c</sup>
Pooled SEM	6.1	3.0

HAMS, high-amylose maize starch; HTHAMS, hydrothermally treated HAMS. Mean values for treatment were significantly different from control: <sup>ab</sup> $P < 0.05$ , <sup>ac</sup> $P < 0.01$ .

For ileal starch digestibility, HTHAMS v. HAMS,  $P = 0.048$ .

For distal colonic starch digestibility, HTHAMS v. HAMS,  $P = 0.027$  and HAMS + HTHAMS v. HAMS,  $P = 0.055$ .

\*Digestibility coefficients were calculated as:

$$\frac{100 - (100 \times \% \text{Cr}_2\text{O}_3 \text{ in diet} \times \% \text{starch in digesta or faeces})}{\% \text{Cr}_2\text{O}_3 \text{ in digesta or faeces}} \times \frac{\% \text{starch in digesta or faeces}}{\% \text{starch in feed}}$$

**Table 4.** Weight (g) and length (m) of the large bowel of pigs fed experimental diets\* (values are least square means and pooled SEM of six animals per group)

Dietary group	Caecum weight	Colon weight			Colon length
		Proximal	Mid	Distal	
Control	61.9 <sup>a</sup>	158.1 <sup>a</sup>	89.5 <sup>a</sup>	104.7 <sup>a</sup>	2.58 <sup>a</sup>
HAMS	126.7 <sup>b</sup>	289.3 <sup>d</sup>	135.5 <sup>d</sup>	133.0	3.07 <sup>c</sup>
HTHAMS	96.7	313.2 <sup>d</sup>	114.5 <sup>d</sup>	156.8 <sup>c</sup>	3.49 <sup>d</sup>
HAMS + HTHAMS	89.8	261.7 <sup>c</sup>	136.5 <sup>d</sup>	146.3 <sup>c</sup>	3.26 <sup>d</sup>
Pooled SEM	18.4	22.7	7.0	9.6	0.10

HAMS, high-amylose maize starch; HTHAMS, hydrothermally treated HAMS. Mean values for treatment were significantly different from control: <sup>ab</sup> $P < 0.05$ , <sup>ac</sup> $P < 0.01$ , <sup>ad</sup> $P < 0.001$ . For distal colon weight, control v. HAMS,  $P = 0.052$  and HTHAMS v. HAMS,  $P = 0.096$ . For colon length, HTHAMS v. HAMS,  $P = 0.007$ . \* Empty wet weight of various compartments and unstretched length of colon.

weight (data not shown). The increase in tissue mass over that of controls was similar in the proximal, mid and distal colon, irrespective of the source of the RS diet (Table 4). Similarly, colon length was increased (by as much as 35%) by all three high-RS diets compared to control (Table 4). Pigs fed diets containing HTHAMS had a longer colon ( $P < 0.01$ ) and tended to have a heavier distal colon ( $P = 0.10$ ) than those fed HAMS. There was a highly significant relationship between estimated RS intake (feed intake  $\times$  ileal starch digestibility) and colon length ( $r^2 = 0.69$ ,  $P < 0.001$ ).

Despite the distinct dietary effects on gross colonic morphology, the microscopic structure of the mid-colonic wall was not markedly affected by feeding diets high in RS (data not shown). Generally, the only linear dimension for mid-colon tissue for which treatment effects were statistically significant was total thickness of the colonic wall, which was greater for HTHAMS than HAMS or HAMS + HTHAMS (2326 v. 1721 and 1887  $\mu\text{m}$ ,  $P < 0.001$  and  $P < 0.05$ , respectively; pooled SEM 116). These values were not significantly different from the controls (1999  $\mu\text{m}$ ).

#### Faecal and large bowel digesta mass and moisture content

Faecal output was significantly greater for pigs fed diets high in RS starch compared to controls (average of 300 g/d in week 2 for the three high-RS diets compared to 103 g/d for controls; pooled SEM 23,  $P < 0.01$ ). Similar differences (approximately twofold) were observed for the amounts of caecal and colonic digesta in pigs fed the control and high-RS diets (Table 5). Values for digesta mass were greatest for HTHAMS, intermediate for HAMS + HTHAMS, and least for HAMS at each large bowel sampling site (same ranking for faecal mass). However, differences between the individual high-RS groups were not uniformly significant across sampling sites. Water content of digesta declined progressively from the ileum to the distal colon (Table 6). Moisture content of ileal digesta was similar among treatment groups ( $P > 0.05$ ) but caecal and proximal colonic digesta of control pigs was drier than that of pigs fed RS diets (Table 6). In addition, digesta in the proximal, mid and distal colon of pigs fed HAMS contained more water per unit mass than that of pigs fed either HTHAMS or HAMS + HTHAMS, and water content of distal colonic digesta was greater for HAMS than controls ( $P < 0.05$ ). Moisture content of faeces collected in week 1 from pigs fed HAMS was greater

( $P < 0.01$ ) than that of controls (67.7/100 g v. 61.4 g/100 g); however, by week 2, faecal water content was reasonably uniform (data not shown) and averaged 65% (range 58–74%) for all groups.

#### Large bowel and faecal pH, and SCFA

Faecal pH was similar (6.39 (pooled SEM 0.05);  $P > 0.05$ ) among the four dietary groups during the adaptive period when all pigs were fed the control diet. pH remained essentially constant in the control group during the subsequent two weeks of study (6.30 and 6.40, respectively, for weeks 1 and 2 of the treatment phase). Feeding HAMS elicited an abrupt and substantial fall of more than 1 pH unit in faecal pH at week 1 (data not shown), and this difference was still evident at week 2 (Table 7). Compared to the control diet, those high in RS resulted in substantially lower digesta pH values throughout the entire large bowel, including the distal colon (Table 7).

Large bowel concentrations of total and major individual SCFA are presented in Fig. 1. Generally, total SCFA concentrations were higher throughout the large bowel, especially in the colon, for pigs fed RS diets compared to controls (Fig. 1(a)). There were few differences in digesta SCFA concentrations between groups fed different types of high-amylose starch. A relative increase in digesta concentrations of acetate and propionate largely accounted for the higher concentrations of total SCFA induced by feeding diets high in RS (Figs. 1(b) and 1(c), respectively). Diets high in RS usually raised digesta butyrate concentration throughout the colon relative to the control diet, but the difference did not always attain statistical significance (Fig. 1(d)). There was no effect of diet on caecal levels of butyrate; however, in the proximal and mid colon, the HAMS treatment elicited a 60% and 120% increase ( $P < 0.05$  and  $P < 0.01$ ), respectively, in digesta butyrate concentrations relative to controls. In the distal colon, only HTHAMS and HAMS + HTHAMS treatments raised butyrate concentration (by 83% and 69%, respectively; both  $P < 0.05$ ).

Large bowel pools of total and individual SCFA were substantially larger in pigs fed diets high in RS (Fig. 2) because of a greater large bowel digesta mass (see Table 5) and higher SCFA concentration (see Fig. 1). Each of the RS diets increased the size of the butyrate pool throughout the entire large bowel, including the distal colon (Fig. 2(d)). The SCFA profile for faeces was similar to that for large bowel digesta

**Table 5.** Wet weight (g) of digesta in the intestinal tract of pigs fed experimental diets (Values are least squares means and pooled SEM of six animals per group except ileum HAMS and HAMS + HTHAMS (*n* 2) and ileum HTAMS (*n* 4))

Dietary group	Ileum	Caecum	Colon			
			Proximal	Mid	Distal	Total colon
Control	25	122 <sup>a</sup>	248 <sup>a</sup>	124 <sup>a</sup>	69 <sup>a</sup>	440 <sup>a</sup>
HAMS	17	306 <sup>d</sup>	556 <sup>d</sup>	277 <sup>d</sup>	170 <sup>b</sup>	1000 <sup>d</sup>
HTHAMS	37	340 <sup>d</sup>	783 <sup>d</sup>	321 <sup>d</sup>	210 <sup>c</sup>	1313 <sup>d</sup>
HAMS + HTHAMS	24	326 <sup>d</sup>	574 <sup>d</sup>	290 <sup>d</sup>	171 <sup>b</sup>	1034 <sup>d</sup>
Pooled SEM	10	31	41	24	27	71

HAMS, high-amylose maize starch; HTHAMS, hydrothermally treated HAMS. Mean values for treatment were significantly different from control: <sup>ab</sup> $P < 0.05$ , <sup>ac</sup> $P < 0.01$ , <sup>ad</sup> $P < 0.001$ . For proximal colon, HTHAMS v. HAMS,  $P = 0.001$  and HTHAMS v. HAMS + HTHAMS,  $P = 0.01$ .

although, unlike digesta, faecal butyrate concentration and molar ratio were not affected by diet ( $P > 0.05$ ; data not shown). Daily faecal excretion of total and major individual SCFA was substantially greater in pigs fed RS diets compared to controls, largely due to a greater faecal mass in the former treatment groups (data not shown). Of the various high-RS dietary treatments, the combination of HAMS and HTHAMS was the most effective at raising faecal SCFA excretion (data not shown).

#### Colonic and faecal microflora

Total anaerobic bacterial density (CFU/g) was greater in proximal colonic digesta in pigs fed the high-RS diets compared to controls (Table 8). The numbers of pigs with colonic bifidobacterial counts below the level of detection of the assay ( $10^4$  CFU/g) was greater for controls than groups on diets high in RS (data not shown). There were no effects of diet on numbers of total aerobic bacteria, although the total anaerobe:aerobe ratio was greater ( $P < 0.05$ ) for pigs fed HAMS compared to controls (data not shown).

In general, there was a progressive decline in faecal counts of viable total aerobes, coliforms and *E. coli*, and an incremental increase in anaerobe:aerobe ratio, as the

**Table 6.** Water content (g/100 g) of digesta in the intestinal tract of pigs fed experimental diets

(Values are least squares means and pooled SEM of six animals per group except ileum HAMS and HAMS + HTHAMS (*n* 2), ileum control (*n* 5) and ileum HTAMS (*n* 4))

Dietary group	Ileum	Caecum	Colon		
			Proximal	Mid	Distal
Control	85.1	83.7 <sup>a</sup>	78.3 <sup>a</sup>	73.1 <sup>a</sup>	68.3 <sup>a</sup>
HAMS	83.0	78.7 <sup>d</sup>	74.6 <sup>c</sup>	74.3	72.0 <sup>b</sup>
HTHAMS	83.1	74.2 <sup>d</sup>	72.1 <sup>d</sup>	71.2	68.1
HAMS + HTHAMS	79.6	76.8 <sup>d</sup>	72.0 <sup>d</sup>	70.2 <sup>b</sup>	67.7
Pooled SEM	2.4	0.6	0.7	0.9	0.9

HAMS, high-amylose maize starch; HTHAMS, hydrothermally treated HAMS. Mean values for treatment were significantly different from control: <sup>ab</sup> $P < 0.05$ , <sup>ac</sup> $P < 0.01$ , <sup>ad</sup> $P < 0.001$ .

For proximal colon, HAMS v. HTHAMS,  $P = 0.03$  and HAMS v. HAMS + HTHAMS,  $P = 0.02$ .

For mid colon, HAMS v. HTHAMS,  $P = 0.02$  and HAMS v. HAMS + HTHAMS,  $P = 0.004$ .

For distal colon, HAMS v. HTHAMS,  $P = 0.009$  and HAMS v. HAMS + HTHAMS,  $P = 0.005$ .

experiment progressed (data not shown). The bacterial population profile of faeces and proximal colonic digesta was similar for each of the treatment groups (Table 8). Density of faecal anaerobes was unaffected by diet, but feeding HAMS or HTHAMS increased ( $P < 0.05$ ) faecal numbers of lactobacilli and bifidobacteria compared to the control diet (Table 8). Faecal counts of coliforms and *E. coli* were lower after 2 weeks of feeding HTHAMS, either alone or in combination with HAMS, compared to controls. However, HAMS as the sole RS in the diet did not affect faecal numbers of these bacterial groups.

#### Discussion

All diets used in the present study contained equal amounts of starch. However, as expected, the amylo maize starches HTHAMS and HAMS were less digestible than the control starch and were therefore much higher in RS. About 12% and 30% of starch present in HAMS and HTHAMS, respectively, resisted small intestinal digestion while the control diet contained <4% RS. The digestibility values for HAMS and also HAMS + HTHAMS are of lesser reliability because of the limited number of observations. It was not possible (for logistic reasons) to collect intestinal contents any earlier than 16 h after the last feeding. This may have contributed to the absence of adequate quantities of terminal ileal digesta in some pigs so the ileal digestibility data should be interpreted with caution. Nevertheless, interpolation of data obtained for HAMS and the mixed RS treatment (HAMS + HTHAMS) indicates that the RS content of HAMS may be in the order of 20%, which is near to the value (30%) reported for the same powdered product incorporated into various cooked foods and eaten by volunteer ileostomates (Muir *et al.* 1994). Govers *et al.* (1999) report that for pigs fed a diet containing coarsely ground high-amylose maize kernels, about 60% of RS was detected in ileal digesta.

It seems that most types of RS tested to date are fermented more or less to completion by the porcine large bowel microflora (Bach Knudsen & Hansen, 1991; Bird *et al.* 2000b; Martinez-Puig *et al.* 2003). Both HAMS and HTHAMS were fermented extensively in this experiment but about 7% and 17%, respectively, appeared in the faecal stream. Faecal starch excretion was greater in this experiment than in a previous study in which pigs were fed diets containing a similar level of HAMS (Brown *et al.* 1997). Pluske *et al.*

**Table 7.** pH of digesta in the large bowel and faeces of pigs fed experimental diets  
(Values are least squares means and pooled SEM of six animals per group)

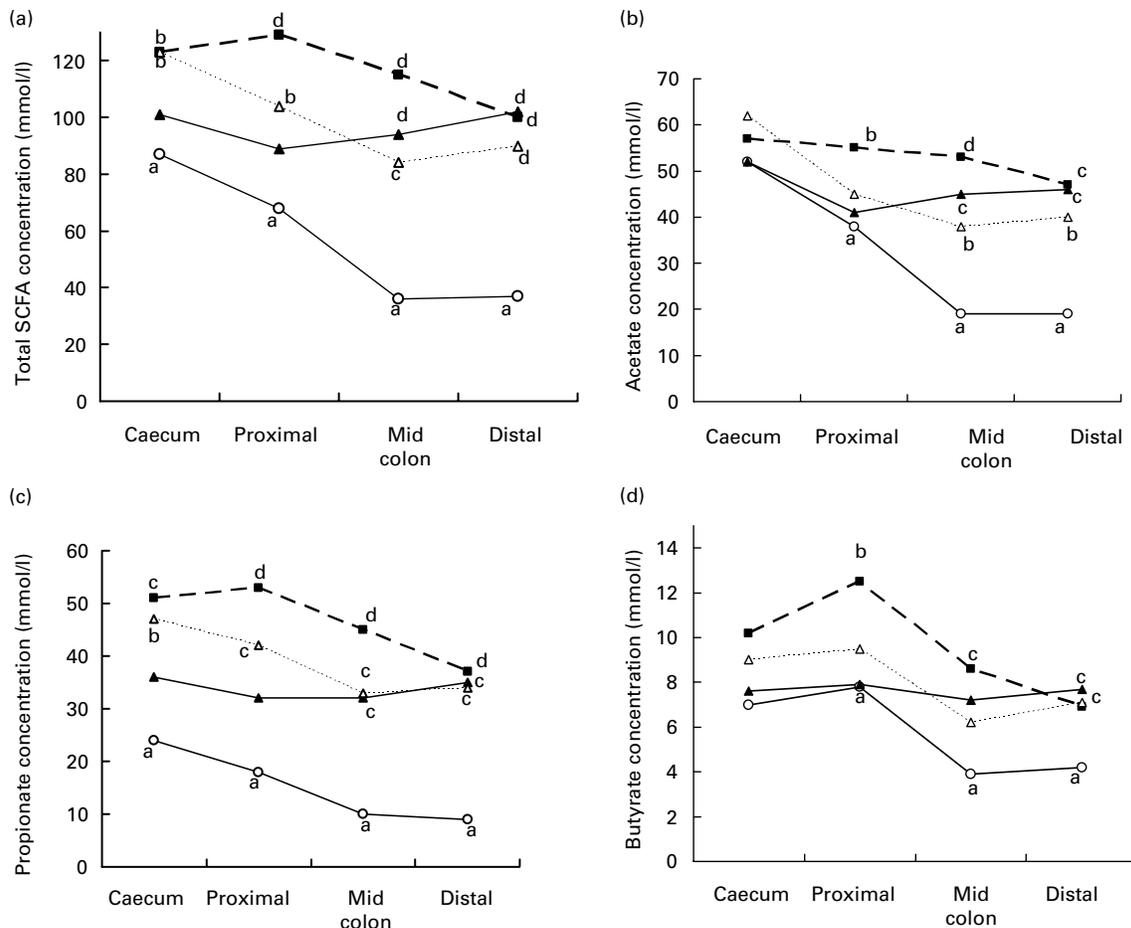
Dietary group	Caecal	Proximal colon	Mid colon	Distal colon	Faeces*
Control	6.58 <sup>a</sup>	6.82 <sup>a</sup>	6.98 <sup>a</sup>	6.75 <sup>a</sup>	6.40 <sup>a</sup>
HAMS	5.31 <sup>d</sup>	5.29 <sup>d</sup>	5.39 <sup>d</sup>	5.52 <sup>d</sup>	5.27 <sup>d</sup>
HTHAMS	5.47 <sup>d</sup>	5.53 <sup>d</sup>	5.42 <sup>d</sup>	5.27 <sup>d</sup>	5.14 <sup>d</sup>
HAMS + HTHAMS	5.43 <sup>d</sup>	5.43 <sup>d</sup>	5.42 <sup>d</sup>	5.36 <sup>d</sup>	5.20 <sup>d</sup>
Pooled SEM	0.08	0.07	0.10	0.11	0.10

HAMS, high-amylose maize starch; HTHAMS, hydrothermally treated HAMS.  
Effects were significant for diet ( $P < 0.001$ ) but not site or their interaction ( $P > 0.05$ ).  
Mean values for treatment were significantly different from control: <sup>ad</sup> $P < 0.001$ .  
For proximal colon, HAMS v. HTHAMS,  $P = 0.03$ .

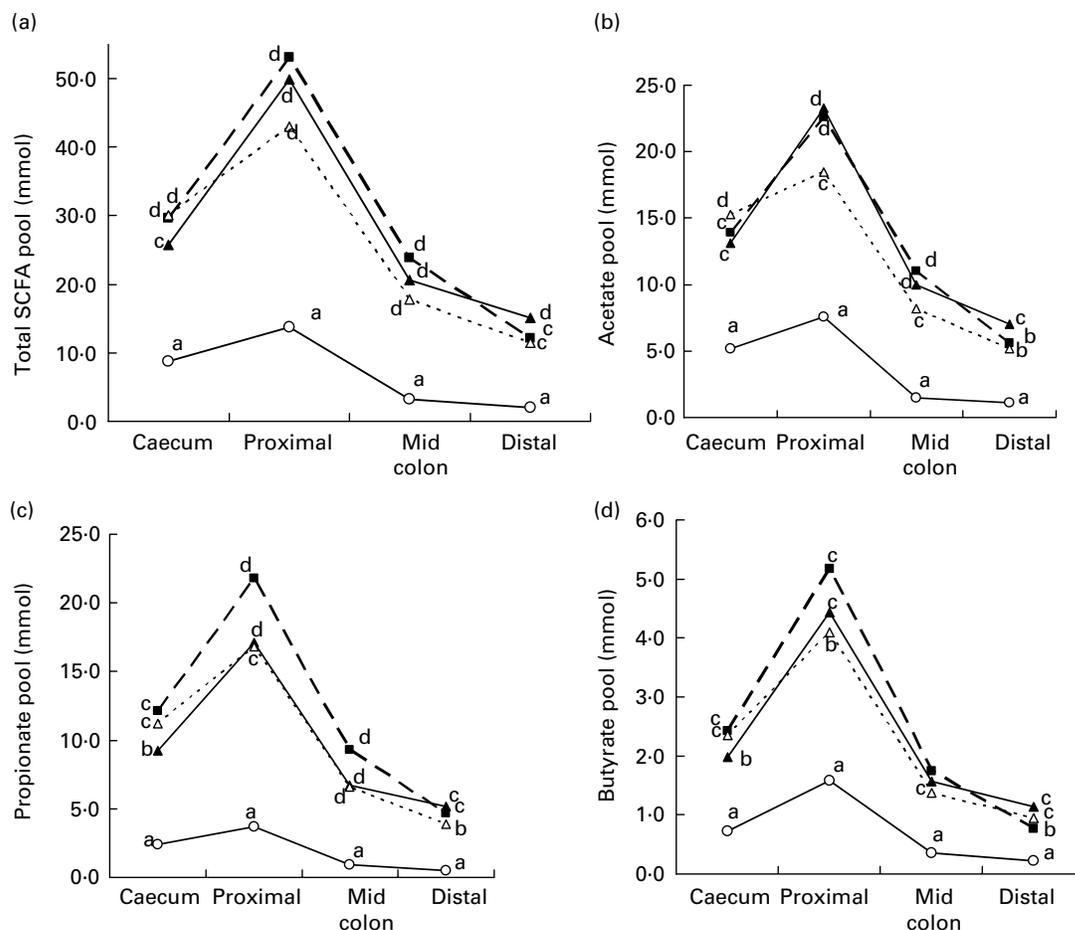
\* Faecal samples were collected on day 14 whereas digesta was sampled on day 21.

(1997) have shown that small intestinal starch digestibility of growing pigs may increase with advancing age and the fact that our pigs were younger than those used by Brown *et al.* (1997) may explain this difference. High-RS diets have also been shown to increase faecal starch excretion in man (Phillips *et al.* 1995), supporting the suggestion that RS fermentation can be less than complete. Although HTHAMS was less digestible in the small bowel than HAMS, these starches exhibited similar large bowel fermentability, with

about 45% of the starch that passed the terminal ileum subsequently disappearing in the large bowel. Accordingly, about 30 g more starch was degraded by the large bowel microflora of pigs fed HTHAMS than HAMS (96 g/d v. 67 g/d, respectively, at the end of experiment). Presumably the much longer retention time of digesta in the large compared to the small intestine (more than about tenfold) explains why whole tract digestibility was similar for the two amylo maize starches but small intestinal digestibility



**Fig. 1.** Concentrations of (a) total and individual SCFA (b–d, acetate, propionate and butyrate, respectively) in caecal, and proximal, mid and distal colonic digesta of pigs fed diets containing low- (Control) or various high-amylose resistant starches (—○—, Control; —■—, high-amylose maize starch (HAMS); —▲—, hydrothermally treated HAMS (HTHAMS); —△—, HAMS + HTHAMS). Values are means for six pigs; pooled SEM values for total, acetate, propionate and butyrate concentrations were 9.1, 5.3, 4.9 and 1.2 mmol/l, respectively. Effect of diet and sampling site were significant for total and each individual SCFA (all  $P < 0.001$ ). Mean values with different letters within a compartment are significantly different from control (<sup>ab</sup> $P < 0.05$ , <sup>ac</sup> $P < 0.01$ , <sup>ad</sup> $P < 0.001$ ).



**Fig. 2.** Pools of total (a) and individual SCFA (b–d, acetate, propionate and butyrate, respectively) in the caecum, and proximal, mid and distal colon of pigs fed diets containing low- (Control) or various high-amylose resistant starches (—○—, Control; —■—, high-amylose maize starch (HAMS); —▲—, hydrothermally treated HAMS (HTHAMS); —△—, HAMS + HTHAMS). Values are means for six pigs; pooled SEM values for total, acetate, propionate and butyrate pools were 3.4, 1.8, 1.6 and 0.4 mmol, respectively. Effect of diet and site were significant for total and each individual SCFA (all  $P < 0.001$ ). Diet  $\times$  site interaction for total SCFA pool ( $P < 0.01$ ). Mean values with different letters within a compartment are significantly different from control ( $^{ab}P < 0.05$ ,  $^{ac}P < 0.01$ ,  $^{ad}P < 0.001$ ).

was not. As mentioned previously, comparisons should be interpreted cautiously given the small number of estimates of ileal digestibility for HAMS (and the combined maize starch treatment).

Large bowel digesta mass and faecal output were increased by feeding amylo maize starches. The contents of all large bowel compartments were numerically greater for HTHAMS than HAMS but were not significant statistically.

**Table 8.** Viable bacterial counts ( $\log_{10}$  CFU/g) in the proximal colonic digesta and faeces (Values are least squares means and pooled SEM of six animals per group)

Sample source and dietary group	Total anaerobes	Total aerobes	Coliforms	<i>E. coli</i>	Lactobacilli	Bifidobacteria
<b>Digesta</b>						
Control	8.73 <sup>a</sup>	7.79	7.79	7.65	6.63 <sup>a</sup>	4.50 <sup>a</sup>
HAMS	9.70 <sup>d</sup>	7.62	7.57	7.08	7.50 <sup>b</sup>	8.12 <sup>c</sup>
HTHAMS	9.49 <sup>d</sup>	7.68	7.52	7.18	7.70 <sup>b</sup>	8.12 <sup>c</sup>
HAMS + HTHAMS	9.56 <sup>d</sup>	7.87	7.74	7.64	7.22	6.79 <sup>b</sup>
Pooled SEM	0.12	0.33	0.34	0.37	0.27	0.68
<b>Faeces*</b>						
Control	9.09	7.99 <sup>a</sup>	7.97 <sup>a</sup>	7.80 <sup>a</sup>	5.94 <sup>a</sup>	5.56 <sup>a</sup>
HAMS	9.47	7.89	7.80	7.42	7.96 <sup>c</sup>	8.45 <sup>c</sup>
HTHAMS	9.37	7.41	7.29	6.73 <sup>b</sup>	7.52 <sup>b</sup>	7.89 <sup>b</sup>
HAMS + HTHAMS	9.55	7.16 <sup>b</sup>	6.94 <sup>b</sup>	6.51 <sup>b</sup>	8.06 <sup>c</sup>	7.63
Pooled SEM	0.22	0.25	0.34	0.27	0.43	0.71

CFU, colony forming units; HAMS, high-amylose maize starch; HTHAMS, hydrothermally treated HAMS. Mean values for treatment were significantly different from control:  $^{ab}P < 0.05$ ,  $^{ac}P < 0.01$ ,  $^{ad}P < 0.001$ .

\* Samples collected on week 2 of the treatment period.

The actual amount of RS was insufficient to account fully for the large bowel digesta mass in pigs fed RS. Presumably, the increase in digesta weight must come mostly from biomass (Shetty & Kurpad, 1986; Cummings *et al.* 1996), which is consistent with the greater number of culturable bacteria in the colon of pigs on the amylo maize diets. The amylo maize diets also may have increased digesta mass by sparing some NSP from fermentation (Phillips *et al.* 1995; Cummings *et al.* 1996), possibly by down-regulating microbial  $\beta$ -glucosidase activity (Hylla *et al.* 1998). However, assuming that this mechanism operates in pigs, its contribution is likely to be small (Govers *et al.* 1999). Previous studies have shown increases in digesta and faecal weight in pigs with feeding of amylo maize (e.g. Brown *et al.* 1997) and other (e.g. Bird *et al.* 2000b) types of RS. RS consumption has been shown to increase faecal weight in most human studies (Phillips *et al.* 1995; Cummings *et al.* 1996; Noakes *et al.* 1996), but the effect is relatively modest. In the present study, the increase in digesta and faecal mass depended on the type of starch, with HTHAMS more effective than HAMS but only in the proximal colon (see Table 5).

Fermentation was increased markedly by inclusion of amylo maize starch in the diet as evidenced by the greater concentrations and pools of SCFA throughout the large bowel and faeces. Similar data have been reported previously for pigs (Govers *et al.* 1999) and other species, including rats (Coleman *et al.* 2002). Other forms of RS (e.g. RS in cooked and cooled brown rice; Bird *et al.* 2000b) have given similar outcomes. Amylo maize also stimulates colonic fermentation in human subjects as evidenced by increased faecal SCFA levels (Noakes *et al.* 1996). As expected, SCFA concentrations were greatest in the proximal colon and declined towards the distal region. This spatial distribution is similar to that seen in human subjects (Mitchell *et al.* 1985) and is thought to reflect the progressive depletion of fermentative substrate and the rapid absorption of SCFA by the epithelium on passage of the faecal stream (Cummings, 1981).

The amylo maize starch diets altered the pattern of fermentation with a higher molar contribution by propionate that occurred largely at the expense of acetate. These changes have been noted previously with HAMS in the large bowel and faeces of pigs (Topping *et al.* 1997) and with other sources of RS, such as legumes (Fleming *et al.* 1989) and cooked brown rice (Bird *et al.* 2000b). Butyrate levels were also higher in pigs fed RS but the effects were less in the distal colon. There is a body of *in vitro* and *in vivo* experimentation to support the view that starch fermentation favours butyrate production compared with other carbohydrate substrates (Englyst *et al.* 1987; Topping & Clifton, 2001). Butyrate is a major metabolic fuel for normal colonocytes and is a key regulator of their proliferation, differentiation and apoptosis (Sengupta *et al.* 2006). The lower level in the distal bowel may reflect preferential butyrate metabolism by the epithelium, which could be of benefit in lowering the risk of serious non-communicable diseases such as colorectal neoplasia, especially in the distal colon, where the incidence of intestinal neoplasms is highest (Rabeneck *et al.* 2003). Although HTHAMS supplied more substrate to the colonic microflora than HAMS, there were no statistically significant

differences between these amylo maizes, either alone or in combination, in increasing butyrate pools (see Fig. 1).

*In vivo* (Topping *et al.* 1997; Govers *et al.* 1999) and *in vitro* experiments (Christl *et al.* 1997) indicate that microbial HAMS fermentation is reasonably rapid. However, in the present study, both HTHAMS and HAMS elicited luminal changes that spanned the complete length of the large bowel and modest quantities of unfermented starch were excreted in faeces. These data indicate that fermentation of these particular starches may not be particularly fast. The RS source, as well as various other factors, including the dietary amount, age of the animal and dietary level, may be important. Other dietary constituents, notably insoluble NSP, can also influence the rate and site of starch fermentation *in situ* (Govers *et al.* 1999). RS types that are not readily fermented might afford a distinct advantage over more susceptible substrates, such as oligosaccharides and soluble NSP, in maintaining the health of the distal bowel. Combinations of RS with various NSP sources have proved effective in moving the site of maximal carbohydrate breakdown further along the gastrointestinal tract of rats (Le Leu *et al.* 2002), pigs (Govers *et al.* 1999) and man (Muir *et al.* 2004).

Consumption of RS had a significant trophic effect on the large bowel, manifest partly as a lengthening of the colon. This action was greater for HTHAMS than HAMS but only HAMS provoked an increase in the weight of the empty caecum. Others have reported increases in gut weight in pigs (Martinez-Puig *et al.* 2003) and rats ingesting RS (Lopez *et al.* 2000; Perrin *et al.* 2001) or other fermentable polysaccharides (Perrin *et al.* 2001; Verghese *et al.* 2002). Raw potato starch (Martinez-Puig *et al.* 2003) and a specific amylo maize starch (Topping *et al.* 1997) have been shown to increase colon length of pigs. In the latter study, colonic elongation was proportional to the amylose content of the diet. The present data confirm and extend those findings and show that the elongation is related positively to the amount of starch reaching the terminal ileum. Presumably, the various SCFA, which are the principal products of RS (and fibre) fermentation in the large bowel, are the trophic stimuli (Topping & Clifton, 2001). However, total wet digesta mass was correlated more strongly to the length of the colon ( $r^2$  0.827,  $P < 0.001$ ) than to the quantity of SCFA present in the lumen ( $r^2$  0.494,  $P < 0.001$ ), which suggests that the mechanical actions of RS may have a greater influence on large bowel size and structure than fermentation-mediated events. However, micromorphometric analysis (of the mid colon) did not demonstrate a thickening of the musculature in response to RS ingestion.

The present investigation provides further evidence that amylo maize starches can act as prebiotics in that they selectively promote the proliferation and/or activity of one or a limited range of colonic bacteria considered beneficial to the health of the host (Gibson & Roberfroid, 1995). HAMS and HTHAMS were equally effective and both increased bifidobacteria counts in the proximal colon by more than 3 log units compared to the control group. The prebiotic effect was not as remarkable when measured in faeces, largely because of the higher bifidobacterial concentrations of the control pigs (see Table 8). A variety of factors have been shown to influence the magnitude of the prebiotic effect, including the initial size of the microbial population targeted by the intervention (Tuohy *et al.*

2001). In the present study, the faecal bifidobacteria population of the control group is comparable to previous reports for pigs fed various diets (Moore *et al.* 1987). Amylomaize has also been shown to be a bifidogenic prebiotic in mice (Wang *et al.* 2002). *In vitro* studies have demonstrated that bifidobacteria is a major starch utiliser (Macfarlane & Englyst, 1986), which is consistent with the *in vivo* effects just mentioned. For most variables, effects were greatest for HTHAMS, intermediate when the two amylomaizes were given together, and least for HAMS. However, in terms of bifidogenicity, the amylomaize combination was not as effective as either of the individual RS sources given alone in raising bifidobacteria counts. The reason for the diminished response is not apparent and further investigation is required to understand the interaction between different starches on the large bowel microbiota.

HAMS and HTHAMS also increased the size of the intestinal lactobacilli population, which agrees with previous reports for HAMS and other types of RS (Kleessen *et al.* 1997; Wang *et al.* 2002). However, the result was not as impressive as that seen for bifidobacteria. Feeding HTHAMS either alone or in combination with HAMS was shown to suppress faecal *E. coli* counts relative to controls, whereas HAMS, which had similar physico-chemical effects in the large bowel, was without effect. We have found that a high-RS diet based on cooked and cooled white rice (combination of RS1 and RS3) dramatically reduced the *E. coli* population in the proximal colon of young pigs (A Bird, M Jackson, R Rankin and D Topping, unpublished results).

The current investigation has shown that both HAMS and HTHAMS are rich sources of RS and their consumption profoundly alters the morphology of the porcine large bowel and the physico-chemical and microbial properties of its contents. Of the amylomaize starches, HTHAMS delivered substantially more fermentable carbohydrate to the large bowel than HAMS but this did not necessarily equate to a more active fermentation. Both starches elicited changes in the luminal environment that encompassed the entire large bowel, but there were indications that HTHAMS acted more distally than HAMS. If these results in pigs can be extrapolated to man, foods containing HTHAMS and/or HAMS may be useful for reducing risk of infectious enteric disease and protecting against degenerative large bowel disorders, particularly in the distal colon.

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